Supporting Information

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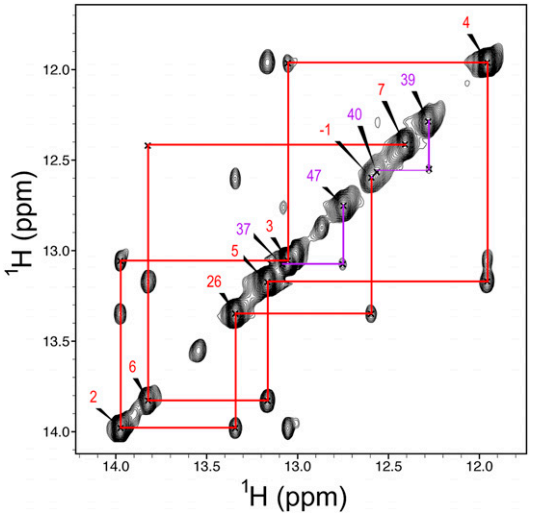

Fig. S1. Imino region of the 2D nuclear Overhauser effect spectroscopy (NOESY) 1-1 echo spectrum at 27 °C of prequeuosine class II (preQ$_1$-II) riboswitch in 60 mM KCl, pH 6.3, on an 800-MHz NMR spectrometer. Red and purple lines show sequential imino connectivities in P2 and P4, respectively.

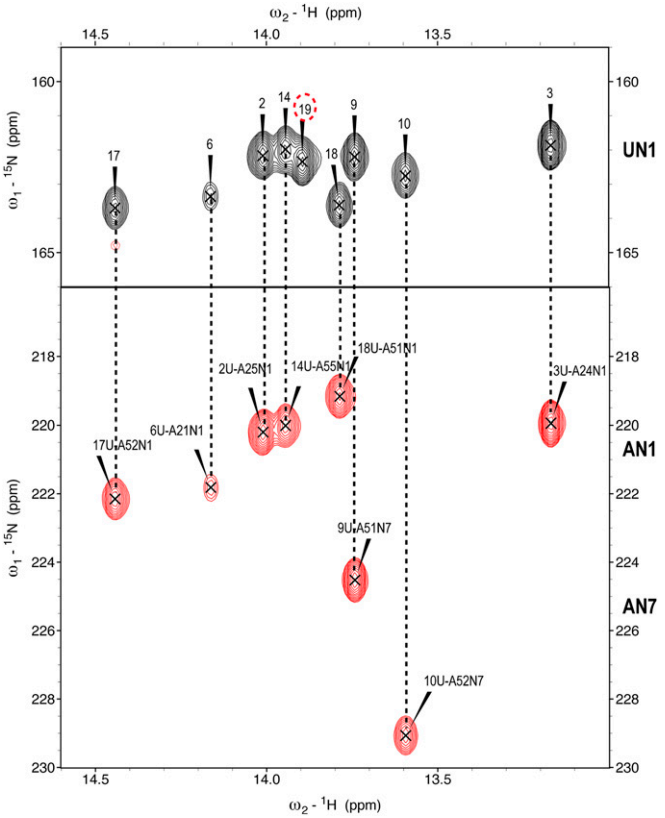

Fig. S2. Pseudo-heteronuclear correlation spectroscopy (J_{NN}-COSY) spectrum of WT preQ$_1$-II riboswitch at 27 °C in 60 mM KCl, 3 mM $CaCl_2$, 2 equivalents of preQ$_1$, pH 6.3, on an 800-MHz NMR spectrometer, showing the correlation between imino protons and corresponding nitrogens in Watson–Crick or Hoogsteen base pairs. No correlation is observed for U19.

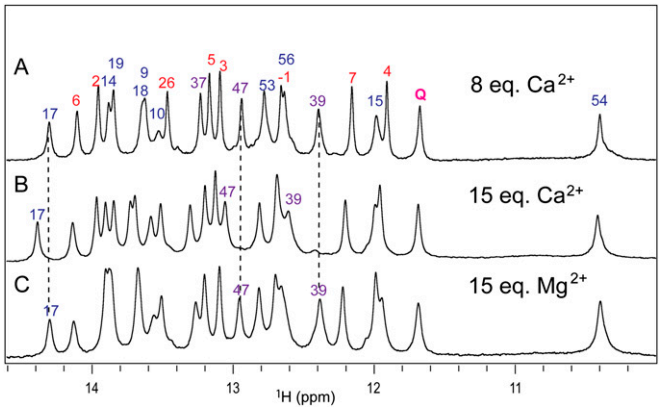

Fig. S3. One-dimensional imino proton spectra at 27 °C of preQ$_1$-II riboswitch in 60 mM KCl, 2 equivalents of preQ$_1$ with 8 equivalents of $CaCl_2$ on 800 MHz (*A*), 15 equivalents of $CaCl_2$ on 600 MHz (*B*), and 15 equivalents of $MgCl_2$ on 600 MHz (*C*). The spectra show that 8 equivalents of Ca^{2+} result in almost the same chemical shifts as 15 equivalents of Mg^{2+}.

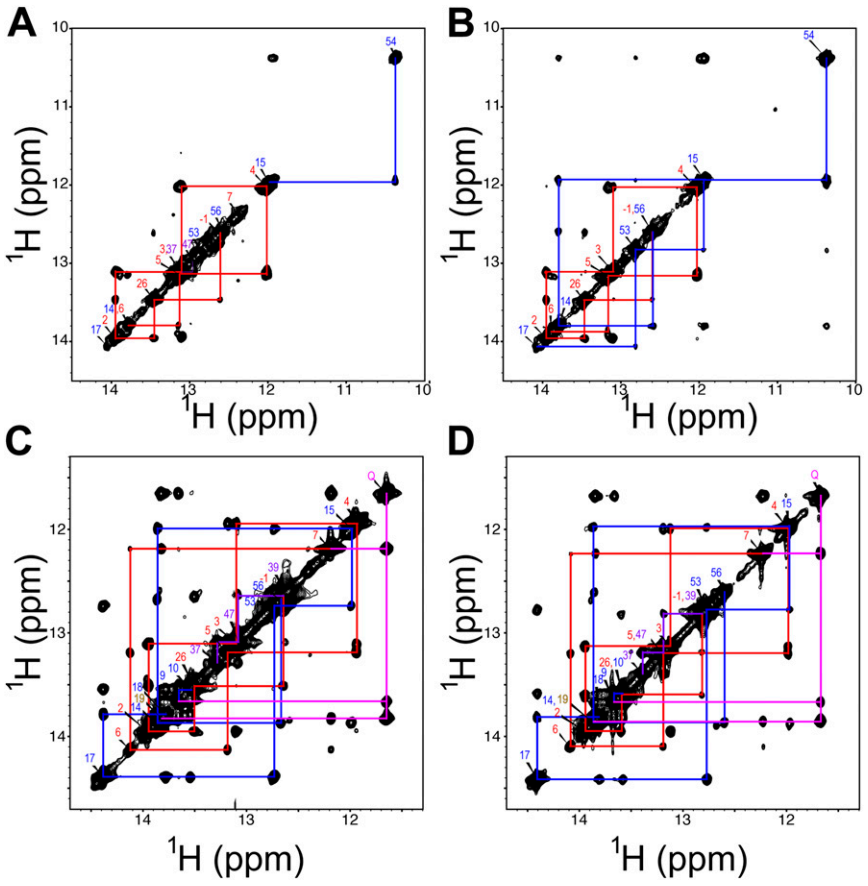

Fig. S4. Imino region of the 2D NOESY 1-1 echo spectrum at 27 °C of WT (*A*) and ΔP4(36-49) preQ$_1$-II riboswitch (*B*) in 60 mM KCl, 4 mM (20 equivalents) $CaCl_2$, pH 6.3, on 800 MHz, and WT (*C*) and (ΔA33C34) preQ$_1$-II riboswitch (*D*) in 60 mM KCl, 3 mM $CaCl_2$, 2 equivalents of preQ$_1$, pH 6.3, on 800 MHz. Red, blue, and purple lines show sequential imino connectivities in P2, P3, and P4, respectively.

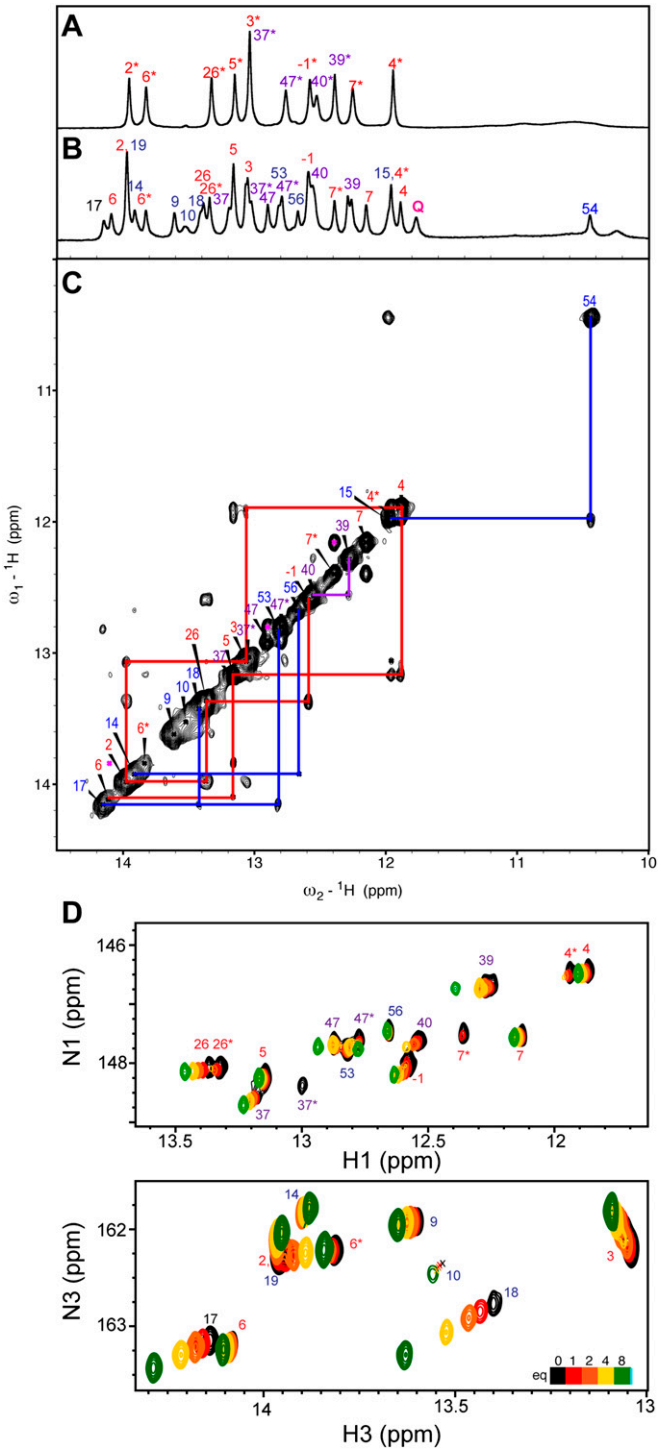

Fig. S5. (*A* and *B*) One-dimensional imino proton spectra at 27 °C of preQ$_1$-II riboswitch in 60 mM KCl (*A*) and 60 mM KCl and 2 equivalents of preQ$_1$ (*B*). (*C*) Imino region of the 2D NOESY 1-1 echo spectrum at 27 °C of *B* on 800 MHz. Red, blue, and purple lines show sequential imino connectivities in P2, P3, and P4, respectively (corresponding to labels in *A* and *B*). (*D*) ^{1}H-^{15}N heteronuclear single-quantum correlation (HSQC) spectra of preQ$_1$-II riboswitch as a function of added Ca^{2+}, from 0 equivalents to 8 equivalents, in 60 mM KCl, 2 equivalents of preQ$_1$, pH 6.3, at 27 °C on 800 MHz. The intensity of the U10 N3-H3 cross-peak is too low to be visible at the contour levels shown at Ca^{2+} below 8 equivalents; its chemical shift positions are marked with an "×".

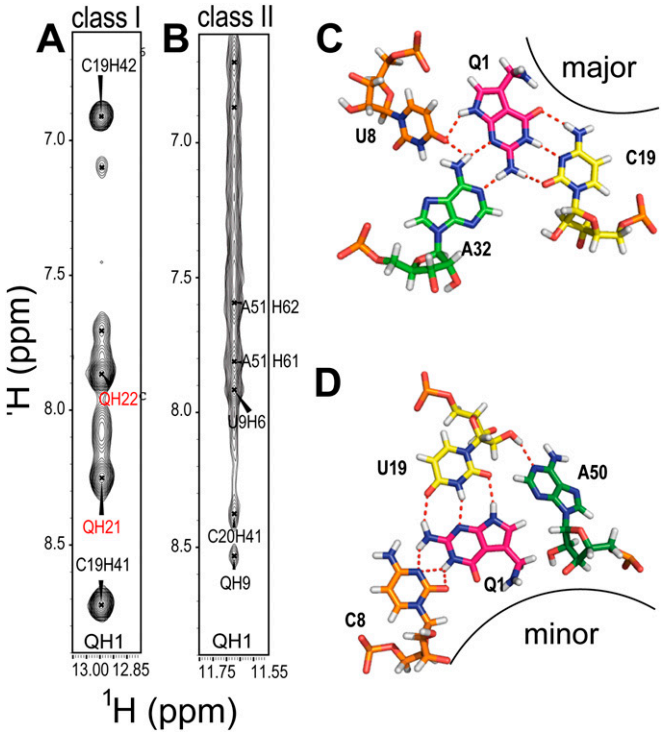

Fig. S6. (*A* and *B*) Slices of nuclear Overhauser effect (NOE) cross-peaks of preQ$_1$ H1 from 2D NOESY 1-1 echo spectra at 27 °C of *Bacillus subtilis queC* (*Bsu*) preQ$_1$-I riboswitch in 50 mM KCl, 2 equivalents of preQ$_1$ (31) (*A*) and *Streptococcus pneumoniae* (*Spn*) preQ$_1$-II riboswitch in 60 mM KCl, 3 mM $CaCl_2$, and 2 equivalents of preQ$_1$ (*B*). (*C* and *D*) PreQ$_1$–base interactions in *Bsu* preQ$_1$-I riboswitch (*C*) and *Spn* preQ$_1$-II riboswitch (*D*).

Table S1. NMR constraints and structure statistics for *Spn* preQ$_1$-II riboswitch (59 nt)

Statistic	Value
Distance constraints	
Total NOE	866
Intraresidue	306
Interresidue	516
Sequential ($\|i - j\| = 1$)	326
Nonsequential ($\|i - j\| > 1$)	190
Intermolecular (preQ$_1$-RNA)	44
Hydrogen bond constraints	122
Total RDCs ($^1D_{CH}$ and $^1D_{NH}$)	103
Total dihedral angle constraints	220
Ribose	59
Glycosidic bond	59
Backbone, based on A-form geometry	102
Structure statistics	
Violations, mean ± SD	
Distance constraints, Å	0.039 ± 0.004
Dihedral angle constraints, °	0.26 ± 0.06
Maximum dihedral angle violation, °	0.36
Maximum distance constraint violation, Å	0.05
Dipolar coupling, Hz	0.59 ± 0.06
Deviations from idealized geometry	
Bond length, Å	0.006 ± 0.0004
Bond angle, °	1.16 ± 0.02
Impropers, °	0.59 ± 0.05
Average pairwise rmsd from mean, Å*	
All heavy atoms (residues in P2, P3, P4, and preQ$_1$)	1.13 ± 0.18
All heavy atoms (residues in P2, P3, and preQ$_1$)	0.95 ± 0.22
preQ$_1$ binding pocket (residues 8–10,17–19,35,50–52, and preQ$_1$)	0.71 ± 0.19
Number of NOE violations >0.5, Å	0.17 ± 0.51
Number of NOE violations >0.2 from P2, P3, and P4, Å	0.11 ± 0.32
Number of dihedral violations >5, °	0.88 ± 0.90
Number of RDC violations >2, Hz	1.38 ± 1.14

*Pairwise rmsd was calculated among 18 refined structures.

Table S2. ITC measurements for *Spn* preQ$_1$-II riboswitch and mutants

RNA	Cation (3 mM)	K_D, nM	N	ΔH, kcal/mol	−TΔS, kcal/mol*	ΔG, kcal/mol†	ΔΔG, kcal/mol‡
WT	None	6,500 ± 700	1.2 ± 0.2	−31 ± 1.6	24 ± 3	−7.1 ± 3.5	2.6
WT	Mg^{2+}	86 ± 3	0.9 ± 0.01	−24 ± 0.1	14 ± 0.1	−9.6 ± 0.1	0.1
WT	Ca^{2+}	75 ± 10	0.9 ± 0.04	−27 ± 0.8	17 ± 0.8	−9.7 ± 0.1	–
P1+WT	Ca^{2+}	220 ± 30	0.6 ± 0.05	−26 ± 3.3	17 ± 3	−9.1 ± 4.7	0.6
ΔP4(36-49)	Ca^{2+}	2,000 ± 300	0.5 ± 0.06	−16 ± 0.6	8 ± 2	−7.8 ± 2.2	1.5
A35G	Ca^{2+}	950 ± 300	0.8 ± 0.0	−17 ± 1.5	9 ± 2	−8.2 ± 2.2	1.9
A50G	Ca^{2+}	24,000 ± 6,000	0.9 ± 0.1	−10 ± 1.6	4 ± 2	−6.3 ± 2.2	3.4
ΔA33C34	Ca^{2+}	190 ± 10	1.1 ± 0.0	−22 ± 0.1	13	−9.2	0.5
ΔA33C34+A35U	Ca^{2+}	900 ± 100	1.2 ± 0.6	−23 ± 4.2	15 ± 4	−8.3 ± 6	1.5

* −TΔS was calculated using T = 298.15 K.
†ΔG is reported as Gibbs free energy, ΔG = ΔH − TΔS.
‡ΔΔG is calculated relative to WT in Ca^{2+}.
RNA sequences (modified or deleted residues indicated by either underlines or dashes):
P1+WT:
gguugaaugaaucaacc - CUUGGUGCUUAGCUUCUUUCACCAAGCAUAUUACACGCGGAUAACCGCCAAAGGAGAA
WT:
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUUACACGCGGAUAACCGCCAAAGGAGAA
ΔP4(36-49):
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUUACA - - - - - - - - - - - - - - AAAGGAGAA
A35G:
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUUACGCGCGGAUAACCGCCAAAGGAGAA
A50G:
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUUACACGCGGAUAACCGCCGAAGGAGAA
ΔA33C34:
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUU - -ACGCGGAUAACCGCCAAAGGAGAA
ΔA33C34+A35U:
gCUUGGUGCUUAGCUUCUUUCACCAAGCAUAUU - -UCGCGGAUAACCGCCAAAGGAGAA

Table S3. Table of RDCs

Residue	Atom	RDC, Hz	Residue	Atom	RDC, Hz	Residue	Atom	RDC, Hz
G-1	*C8H8*	*-2.69*	U19*	C1′H1′	−8.58	C38*	C5H5	21.11
G-1	*C1′H1′*	*12.91*	C20*	C6H6	−9.65	G39*	N1H1	7.68
G-1	N1H1	−1.53	C20*	C5H5	23.08	G39	C8H8	−16.13
C1	*C5H5*	*14.16*	C20*	C1′H1′	−8.73	G39*	C1′H1′	14.18
U2*	N3H3	−0.32	A21*	C2H2	−2.28	G40	C1′H1′	1.53
U2*	C5H5	−12.57	A21*	C1′H1′	−12.69	*A41*	*C1′H1′*	*-6.95*
U3*	N3H3	−9.68	C22*	C5H5	−8.54	A43	C8H8	−7.24
U3*	C5H5	−9.1	C22*	C1′H1′	−17.4	*A43*	*C1′H1′*	*-1.33*
U3	*C1′H1′*	*-13.07*	C23*	C5H5	−6.38	*A43*	*C2H2*	*6.83*
G4*	N1H1	−11.71	A24*	C8H8	27.39	*A44*	*C2H2*	*-5.45*
G4*	C8H8	26.05	A24*	C2H2	20.97	C45	C5H5	12.09
G4*	C1′H1′	−8.53	A25*	C8H8	22.11	C46*	C5H5	11.88
G5*	N1H1	−2.61	A25*	C1′H1′	3.33	C46*	C1′H1′	1.21
G5*	C8H8	21.22	G26*	N1H1	4.69	G47*	N1H1	3
G5*	C1′H1′	−3.68	G26*	C8H8	−4.96	G47*	C8H8	−17.58
U6*	N3H3	3.27	G26*	C1′H1′	1.8	G47*	C1′H1′	−8.14
U6*	C5H5	24.55	C27	C6H6	−4.55	*C48*	*C6H6*	*21.43*
G7*	N1H1	2.3	C27	C5H5	5	C48	C5H5	−14.15
G7*	C8H8	−11.66	*A28*	*C2H2*	*-6.29*	*C49*	*C6H6*	*22.65*
C8*	C6H6	−3.82	*A28*	*C1′H1′*	*-10.86*	C49	C5H5	−6.93
C8*	C5H5	−3.93	*U29*	*C6H6*	*10.92*	C49*	C1′H1′	4.84
C8*	C1′H1′	−8.74	*U29*	*C5H5*	*-0.19*	A50*	C8H8	17.25
U9*	N3H3	0.14	*U29*	*C1′H1′*	*-1.39*	A50*	C2H2	−0.45
U9*	C5H5	−5.9	*A30*	*C8H8*	*1.28*	A50*	C1′H1′	−7.68
U10*	N3H3	−9.84	*A30*	*C2H2*	*3.54*	A51*	C1′H1′	−13.55
U10*	C5H5	−11.91	*A30*	*C1′H1′*	*3.49*	A52*	C2H2	23.32
A11*	C8H8	8.14	*U31*	*C6H6*	*4.03*	A52*	C1′H1′	−6.51
A11*	C1′H1′	−7.03	*U31*	*C5H5*	*-1.18*	G53*	N1H1	5.31
G12*	C8H8	2.07	*U31*	*C1′H1′*	*0.3*	G53*	C8H8	−3.16
G12*	C1′H1′	−9.56	*U32*	*C6H6*	*3.39*	G53*	C1′H1′	−6.47
C13*	C5H5	−1.01	*U32*	*C1′H1′*	*0.19*	G54*	N1H1	3.43
C13	*C1′H1′*	*-11.49*	*A33*	*C8H8*	*3.32*	G54*	C8H8	−9.41
U14*	N3H3	−8.1	*A33*	*C2H2*	*1.65*	G54*	C1′H1′	−9.38
U14	*C5H5*	*22.17*	*A33*	*C1′H1′*	*2.55*	A55*	C2H2	−9.19
U15*	N3H3	−2.92	*C34*	*C6H6*	*3.74*	A55*	C1′H1′	−9.19
U15*	C5H5	26.78	*C34*	*C5H5*	*-1.81*	G56	N1H1	−14.15
C16*	C5H5	15.99	*C34*	*C1′H1′*	*1.22*	*G56*	*C8H8*	*24.49*
U17*	N3H3	0.75	A35	C8H8	−10.46	G56	C1′H1′	−6.37
U18*	N3H3	−7.27	A35*	C2H2	−18.2	*A57*	*C8H8*	*25.01*
U18*	C1′H1′	−11.51	A35	C1′H1′	−9.98	A57	C2H2	9.66
U18*	C5H5	−8.45	G37	N1H1	0.11	A57	C1′H1′	6.3
U19*	N3H3	−10.23	G37	C8H8	15.37	*A58*	*C8H8*	*2.81*
U19	C6H6	17.75	C38*	C6H6	−9.88	A58	C2H2	13.92
U19*	C5H5	6.81	C38*	C1′H1′	11.59	A58	C1′H1′	8.67

*RDCs used in GDO analysis.
RDCs not used for final structural refinements are in italic type.

Table S4. Table of R_1 and R_2 values

Residue	Atom	R_1, Hz	R_2, Hz	Residue	Atom	R_1, Hz	R_2, Hz
G-1	C8	1.63 ± 0.03	64.75 ± 0.87	U29	C6	2.17 ± 0.02	42.66 ± 0.36
G-1	C1′	0.96 ± 0.03	46.48 ± 1.33	U29	C5	1.98 ± 0.01	46.95 ± 0.35
C1	C5	1.62 ± 0.03	77.14 ± 1.01	U29	C1′	1.69 ± 0.01	24.92 ± 0.38
C1	C1′	0.85 ± 0.04	44.49 ± 1.64	A30	C8	1.90 ± 0.01	28.92 ± 0.20
U2	C6	1.29 ± 0.07	77.84 ± 2.64	A30	C2	1.78 ± 0.01	21.54 ± 0.30
U2	C5	1.43 ± 0.03	80.42 ± 1.28	U31	C6	2.69 ± 0.01	28.53 ± 0.18
U3	C6	1.42 ± 0.07	74.08 ± 2.45	U31	C5	2.30 ± 0.01	34.25 ± 0.22
U3	C5	1.50 ± 0.03	83.89 ± 1.35	U31	C1′	1.91 ± 0.01	20.12 ± 0.23
U3	C1′	0.97 ± 0.03	48.18 ± 1.51	U32	C6	2.68 ± 0.01	29.81 ± 0.18
G4	C8	0.80 ± 0.04	60.83 ± 1.40	U32	C5	2.31 ± 0.01	32.95 ± 0.21
G4	C1′	0.94 ± 0.03	46.48 ± 1.50	U32	C1′	1.86 ± 0.01	20.89 ± 0.22
G5	C8	0.86 ± 0.04	60.90 ± 1.34	A33	C8	2.02 ± 0.01	28.67 ± 0.22
G5	C1′	0.92 ± 0.03	42.04 ± 1.25	A33	C2	1.98 ± 0.01	34.84 ± 0.15
U6	C6	0.96 ± 0.04	80.11 ± 3.49	A33	C1′	1.78 ± 0.02	22.67 ± 0.35
U6	C5	1.63 ± 0.04	73.98 ± 1.39	C34	C6	2.43 ± 0.02	43.16 ± 0.33
U6	C1′	0.96 ± 0.04	44.79 ± 1.85	C34	C5	2.16 ± 0.01	44.74 ± 0.34
G7	C8	0.84 ± 0.04	63.20 ± 1.51	C34	C1′	1.80 ± 0.02	27.39 ± 0.54
G7	C1′	0.86 ± 0.03	46.40 ± 1.59	A35	C8	1.07 ± 0.04	65.55 ± 1.34
C8	C6	1.41 ± 0.08	80.64 ± 3.11	A35	C2	1.34 ± 0.05	78.15 ± 2.07
C8	C5	1.45 ± 0.03	85.36 ± 1.64	A35	C1′	0.92 ± 0.06	48.22 ± 2.31
C8	C1′	0.85 ± 0.06	43.03 ± 2.91	G37	C8	1.03 ± 0.04	60.73 ± 1.29
U9	C5	1.45 ± 0.05	81.39 ± 2.07	G37	C1′	1.00 ± 0.03	49.69 ± 1.58
U9	C1′	0.94 ± 0.04	48.01 ± 2.09	C38	C6	1.57 ± 0.06	77.85 ± 1.97
U10	C6	1.42 ± 0.09	84.93 ± 3.46	C38	C1′	0.93 ± 0.05	41.07 ± 0.86
U10	C5	1.37 ± 0.08	85.07 ± 3.51	G39	C8	1.01 ± 0.04	64.12 ± 1.34
U10	C1′	0.83 ± 0.08	44.28 ± 3.61	G39	C1′	0.97 ± 0.02	38.76 ± 0.85
A11	C8	1.39 ± 0.07	46.00 ± 1.89	G40	C1′	1.04 ± 0.04	39.65 ± 1.54
A11	C1′	1.14 ± 0.05	46.45 ± 2.11	A41	C1′	1.10 ± 0.02	42.80 ± 0.98
G12	C8	1.13 ± 0.03	55.96 ± 0.80	A43	C2	1.14 ± 0.02	68.15 ± 0.66
G12	C1′	1.06 ± 0.03	43.75 ± 1.47	C45	C6	1.65 ± 0.06	72.33 ± 2.12
C13	C6	1.50 ± 0.08	77.44 ± 2.69	C45	C5	1.76 ± 0.02	64.84 ± 0.86
C13	C5	1.54 ± 0.04	85.31 ± 1.73	C46	C5	1.67 ± 0.03	73.31 ± 1.04
C13	C1′	0.96 ± 0.02	42.71 ± 2.38	C46	C1′	1.01 ± 0.02	41.81 ± 0.87
U15	C6	1.26 ± 0.08	82.15 ± 3.22	G47	C8	1.08 ± 0.03	57.71 ± 0.88
U15	C5	1.52 ± 0.04	78.91 ± 1.60	G47	C1′	0.99 ± 0.02	41.69 ± 0.90
C16	C5	1.52 ± 0.04	79.77 ± 1.77	C48	C1′	0.93 ± 0.02	42.17 ± 0.78
U18	C1′	0.92 ± 0.04	46.44 ± 1.61	C49	C6	1.52 ± 0.05	70.59 ± 1.88
U19	C5	1.59 ± 0.04	81.01 ± 1.64	C49	C5	1.60 ± 0.02	73.93 ± 1.00
U19	C1′	1.02 ± 0.07	42.07 ± 3.07	C49	C1′	1.05 ± 0.03	40.81 ± 1.02
C20	C6	1.34 ± 0.10	80.28 ± 3.69	A50	C8	0.98 ± 0.04	55.33 ± 1.23
C20	C5	1.68 ± 0.04	70.13 ± 1.39	A50	C2	0.96 ± 0.05	74.72 ± 1.56
C20	C1′	0.81 ± 0.05	49.34 ± 2.96	A50	C1′	0.94 ± 0.06	45.48 ± 3.05
A21	C8	0.89 ± 0.03	63.25 ± 0.98	A51	C8	1.50 ± 0.09	62.29 ± 1.92
A21	C2	0.88 ± 0.03	74.42 ± 1.48	A51	C2	0.89 ± 0.02	72.33 ± 0.89
A21	C1′	0.89 ± 0.04	45.18 ± 1.93	A51	C1′	0.72 ± 0.07	48.95 ± 3.01
C22	C6	1.56 ± 0.06	76.68 ± 2.03	A52	C8	0.97 ± 0.03	59.65 ± 1.30
C22	C5	1.50 ± 0.05	88.24 ± 2.17	A52	C2	0.89 ± 0.02	72.33 ± 0.89
C22	C1′	0.90 ± 0.03	48.35 ± 1.80	A52	C1′	0.89 ± 0.05	49.28 ± 2.81
C23	C5	1.50 ± 0.04	81.47 ± 1.62	G53	C8	0.90 ± 0.08	62.41 ± 2.76
A24	C8	1.03 ± 0.04	56.87 ± 1.25	G53	C1′	0.93 ± 0.03	44.43 ± 1.47
A24	C2	0.99 ± 0.03	69.96 ± 0.91	G54	C8	0.86 ± 0.05	66.21 ± 1.73
A24	C1′	0.97 ± 0.03	43.79 ± 1.41	G54	C1′	0.91 ± 0.03	45.42 ± 1.59
A25	C8	1.03 ± 0.03	61.63 ± 1.10	A55	C8	0.89 ± 0.03	63.25 ± 0.98
A25	C2	1.01 ± 0.02	67.27 ± 0.79	A55	C2	0.89 ± 0.03	76.10 ± 1.44
A25	C1′	1.03 ± 0.03	40.57 ± 1.30	A55	C1′	0.92 ± 0.04	43.61 ± 1.48
G26	C8	0.91 ± 0.04	61.21 ± 1.49	G56	C8	0.77 ± 0.05	60.46 ± 1.59
G26	C1′	0.96 ± 0.04	43.03 ± 1.26	G56	C1′	0.92 ± 0.03	39.60 ± 1.65
C27	C6	1.45 ± 0.06	81.17 ± 2.30	A57	C8	1.07 ± 0.03	53.27 ± 0.96
C27	C5	1.56 ± 0.02	75.92 ± 0.93	A57	C1′	1.09 ± 0.02	36.28 ± 0.86
A28	C2	1.12 ± 0.02	65.79 ± 0.51	A58	C8	1.42 ± 0.01	39.88 ± 0.40
A28	C1′	1.16 ± 0.02	38.24 ± 0.75	A58	C2	1.55 ± 0.02	43.29 ± 0.30
				A58	C1′	1.68 ± 0.01	22.01 ± 0.33